SHORT REPORT

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Radical Scavenging and DNA Cleavage Inhibitory Activities of 2,3-Dihydroxybenzoyl Glycine Obtained from *Bacillus subtilis*

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Abstract: A catecholate type of iron chelator (siderophore); 2,3-dihydroxybenzoyl glycine (DHBG) was produced by *Bacillus* sp. under iron stress conditions. Pure DHBG was subjected for DPPH (α,α−Diphenyl−β−Picrylhydrazyl) radical scavenging activity and radical induced DNA cleavage inhibition assay. In results, DHBG showed the highest radical scavenging effect and DNA cleavage inhibition activity when it was free from iron. This study revealed antioxidative potential of iron chelator DHBG; and its probable mechanism.

Keywords: Catechol; 2,3-dihydroxybenzoyl glycine; *Bacillus* sp.; DPPH radical; Iron.

1. Source

The iron chelator 2,3-dihydroxybenzoyl glycine (DHBG) was obtained from *Bacillus subtilis* ST13 as per the method described in our previous report; Ahire *et. al.*,[1]. In brief, *Bacillus subtilis* ST13 (3% v/v) was inoculated in chemically defined low iron medium (CDLIM) and incubated for 36 h with 120 rpm at 37°C. Further, the compound was extracted with ethyl acetate followed by its purification using XAD-4 column chromatography to its homogeneity. The lyophilized compound was characterized and used further in these experiments.

2. Previous Studies

Under iron deficient condition, aerobic organisms produce organic iron chelating compounds known as siderophores to make iron soluble [2-3]. These are various low molecular weight (< 1000 Da) small peptide molecules like catecholates, hydroxamates, a hydroxycarboxylates or mixed molecules bearing ability to chelate ferric ions [1]. May *et. al.*, [4] have reported genus *Bacillus* to produce catecholate type of iron chelator DHBG; where its aromatic hydroxyl groups contribute to iron chelation [4-1].

In this study, DHBG of *Bacillus* sp. demonstrated to possess potent antioxidative activity. The experiments also suggested that, probably the hydroxyl groups of DHBG contribute in free radical scavenging activity, but only when it was free from iron.

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3. Present Study

DPPH radical scavenging activity of DHBG was analyzed by using the method described by Ahire *et. al.,* [1]. In brief, a 0.8 mL of DHBG bearing specific concentration and 1 mL of freshly prepared 0.2 mM DPPH (Sigma) solution in methanol were mixed together to react for 30 min in the dark. Blank was prepared containing only pure methanol. The scavenged DPPH was then monitored by measuring the decrease in optical density at 517 nm. In view of mechanistic investigations, an experiment was carried out in the presence of $FeCl_3$ and EDTA.

DHBG-DPPH reaction was characterized by using a spectrophotometer (UV-Vis 1601, Shimadzu, Japan). The spectrum was recorded in the range of 580 – 190 nm at room temperature in pure methanol. The effect of iron in CAS (Chrome Azurol S) reagent on DHBG-DPPH reaction product was studied.

Furthermore, HPLC (High Performance Liquid Chromatography) analysis of DHBG-DPPH reaction was performed on HPLC (LC-8A Shimadzu, Japan) with gradient pump, Eurospher C_{18} reverse phase column (100-5, 250×4.6 mm with pre-column) as stationary phase, equipped with detector (220 nm), interrogator (Prominence processor) and methanol as mobile phase with a flow rate of 1 ml min^{-1} (programmed for 10 min).

Radical induced DNA cleavage inhibition was performed by treating 300 ng of pBR322 super coiled DNA with different concentrations of DHBG (5-10 µM) in presence of radicals at 37°C for 30 min. The Fenton reaction (equal volume of 0.75 mM $FeSO_4.7H_2O$ and 0.001 % H_2O_2) was used to generate hydroxyl radicals. The superoxide radicals were generated by 10 mM paraquat (1, 1′ -Dimethyl-4, 4′ -bipyridinium dichloride) solution in total 10 µL reaction volume. The pre-incubated reaction mixture was electrophoressed on 1 % agarose gel in tris borate EDTA buffer (TBE pH 8.2) for 5 h at 50 mV. The gel was stained with a 0.5 µg mL^{-1} ethidium bromide and visualized under UV light and photographed for analysis. For mechanistic investigations, experiments were carried out in presence of different combinations of DHBG and EDTA.

DHBG of high purity was brown colored powder having molecular formula $C_9H_9NO_5$ and molecular weight 211.05 [1]. The DPPH radical scavenging activity of DHBG is depicted in Table 1. The effect of iron ($FeCl_3$) on DHBG antioxidant activity was further investigated by reacting both in equimolar concentrations. The radical scavenging of DHBG was drastically decreased to negative value indicating inhibitory effect of iron on antioxidant activity. This has lead to the clue for probable mechanism of radical scavenging. In the view of these results, DHBG was analyzed further for presence of contaminated iron by using atomic absorption spectrophotometer (S-2, Thermo Electron Corporation, USA). It was found that, purified DHBG contains 1.257 µg of iron per mg of DHBG which could be responsible for low values of DPPH radical scavenging activity (Table 1).

However, this inhibitory effect of iron was found to be neutralized by the addition of equimolar concentration of EDTA, since its stability constant is ~25 which is very high conferring strong ability to chelate iron [5]. The values of radical scavenging by DHBG (EDTA treated) were equivalent with ~4 µM ascorbate as compared to untreated DHBG. The EDTA alone showed no radical scavenging activity due to absence of Ar-OH groups.

Table 1. DPPH radical scavenging activity of DHBG under various conditions

Conc. (µM)	The % DPPH radical scavenging activity					
	DHBG	DHBG + $FeCl_3$ (1:1)	EDTA treated DHBG	Ascorbate	EDTA	EDTA + $FeCl_3$ (1:1)
1	5 ± 0.26	− 15.2 ± 0.05	29.3 ± 0.10	15.5 ± 0.10	− 4.5 ± 0.34	− 5.0 ± 0.05
2	5.5 ± 0.10	− 18.3 ± 0.20	30.1 ± 0.20	24.8 ± 0.05	− 5.7 ± 0.11	− 5.7 ± 0.20
3	5.7 ± 0.05	− 22.3 ± 0.11	31.3 ± 0.05	26.1 ± 1.50	− 6.6 ± 0.37	− 7.8 ± 0.40
4	6.2 ± 0.15	− 23.3 ± 0.17	31.9 ± 0.11	34.2 ± 0.50	− 7.5 ± 0.11	− 8.0 ± 0.05

% radical scavenging effect was defined as [O.D. blank− O.D. test / O.D. blank] × 100; each value is the mean of three experiments, ± std. dev

The presence of aromatic OH groups in DHBG mainly contributes toward iron chelation [4] where deprotonation of these groups make them reactive strongly with iron. Considering this fact, we propose a probable mechanism of radical scavenging by DHBG. The deprotonation of aromatic OH group is a key step in reactions of radical stabilization as well as iron chelation. When iron is present in medium it reacts strongly with Ar-OH of DHBG, leaving Ar-OH groups unavailable for scavenging of DPPH radical. In the absence of iron in medium, the 2nd position Ar-OH group of DHBG is deprotonated first being closer to C=O group of the compound. The C=O group leads to more acidic environment and make the hydrogen more positive as compared to hydrogen present at 3rd position of aromatic ring. The release of more positive hydrogen stabilizes DPPH radical and forms 3-hydroxyl-2-phenoxide-1-benzoylglycine (Figure 1).

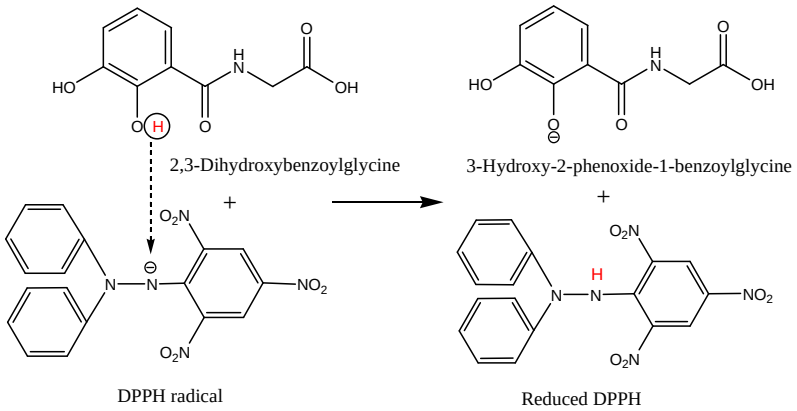

Figure 1. Proposed mechanism of radical scavenging by DHBG

The evidences of spectrophotometric and HPLC analysis support the speculation of the proposed mechanism of radical scavenging by DHBG. The shift of peaks (Figure 2) in spectrophotometric analysis in the range of 580-190 nm indicates radical scavenging by DHBG as well as iron chelation by 3-hydroxyl-2-phenoxide-1-benzoylglycine. The decrease of peak height at 220 nm in HPLC analysis (Figure 3) of DHBG in DHBG-DPPH reaction states the formation of proposed compound; 3-hydroxyl-2-phenoxide-1-benzoylglycine (HPBG). The presence of oxygen radical attached to the aromatic ring in HPBG does not make it unsafe since it gets stabilized rapidly by resonance.

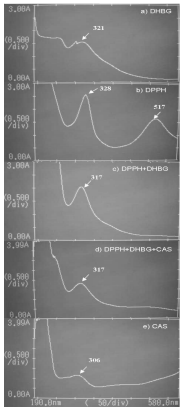

Figure 2. Spectrophotometric analysis of DHBG along with different reactant. a) DHBG in methanol; b) DPPH in methanol; c) DHBG + DPPH mix after 30 min reaction; d) DHBG + DPPH mix after 30 min reaction + CAS reagent (1:1); e) CAS reagent.

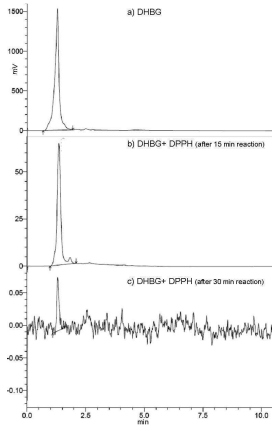

Figure 3. HPLC analysis of DHBG-DPPH reaction. Chromatogram a) DHBG before reaction with DPPH; b) after 15 min of DHBG-DPPH reaction; c) after 30 min of DHBG-DPPH reaction.

The HPBG; a product of DHBG-DPPH reaction was evaluated for iron chelation ability. The HPBG when mixed with blue colored CAS reagent in equal quantities, it produced pink to red color.

The change in color of CAS reagent from blue to pink or red indicates loss of iron from weak chelator present with metal dye, which confirmed the chelation ability of compound [6]. This was clarified and supported further by spectrophotometric analysis (Figure 2).

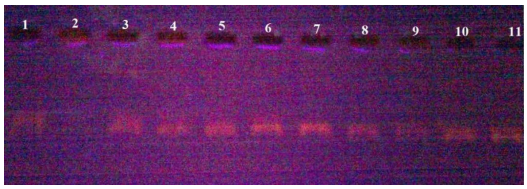

Figure 4. Inhibition of radical induced DNA cleavage activity. Lane: 1: Plasmid + hydroxyl radicals; 2: Plasmid + superoxide radicals; 3: Plasmid + equimolar concentrations of [(10 µM) DHBG+ (10 µM) EDTA] + hydroxyl radicals; 4: Plasmid + equimolar concentrations of [(10 µM) DHBG+ (10 µM) EDTA] + superoxide radicals; 5: Plasmid + (10 µM) DHBG+ hydroxyl radicals; 6: Plasmid + (5 µM) DHBG+ hydroxyl radicals; 7: Control plasmid; 8: Plasmid + (10 µM) DHBG+ superoxide radicals; 9:Plasmid + (5 µM) DHBG+ superoxide radicals; 10: Plasmid + equimolar concentrations of (10 µM DHBG + 10 µM EDTA); 11:Plasmid + (10 µM) DHBG.

In radical induced DNA cleavage inhibition activity (Figure 4), EDTA treated DHBG showed inhibition of DNA cleavage by superoxide radicals and hydroxyl radicals which further supports the antioxidant potential of iron chelators DHBG.

This study revealed the role of iron chelator; DHBG as an antioxidant when it was free from iron. This demonstrated the possible mechanism and applicability of such natural iron chelator in antioxidant therapy.

Acknowledgments

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